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(57) Abstract

"Transit peptides" are known which direct nuclear-encoded chloroplastidic proteins to the chloroplast. Corresponding mitochondrial transit peptides are also known. The invention provides transit peptides which direct nuclear-encoded proteins to both the chloroplast and the mitochondrion. The prototype is derived from glutathione reductase form pea (*Pisum sativum L.*). A DNA sequence encoding a fusion protein of the transit peptide fused to a protein of interest can be used to generate transgenic plants.

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PLANT GENETIC MANIPULATION

This invention relates to plant molecular biology. In particular, it relates to the targeting of plant organelles by chimeric preproteins.

The N-terminal pre-sequences of certain plant proteins are known to be able to target the protein either to the chloroplast or to the mitochondrion, with varying degrees of efficiency. These pre-sequences, which are known as 'transit peptides' or 'targeting sequences', are necessary for nuclear-encoded chloroplastidic or mitochondrial proteins and are generally cleaved from the mature protein during or after translocation into the organelle. Examples of transit peptides known to be chloroplast-specific include N-terminal pre-sequences derived from:

the small subunit of ribulose-1,5-bisphosphate carboxylase, otherwise known as rubisco (Schreier *et al.*, *The EMBO Journal* **4**(1) 25-32 (1985), Van den Broeck *et al.* *Nature* **313** 358-363 (1985) and Guerineau *et al.* *Nucleic Acids Research* **16**(23) 11380 (1988));

the chlorophyll a/b binding proteins (Kavanagh *et al.*, *Mol. Gen. Genet* **215** 38-45 (1988)); and

glutathione reductase (Creissen *et al.* *The Plant Journal* **2**(1) 129-131 (1991).

Examples of transit peptides known to be mitochondrion-specific include N-terminal pre-sequences derived from:

the β-subunit of mitochondrial ATP synthase (Boutry *et al.*, *Nature* **328** 340-342 (1987)); and

mitochondrial tryptophanyl-tRNA synthetase (Schmitz & Lonsdale *The Plant Cell* **1** 783-791 (1989)).

By the use of recombinant DNA encoding proteins which are a fusion between one of the above transit peptides and a protein of interest it is possible to target the protein of interest to the chloroplast or mitochondrion. However, it is generally accepted in the art, as represented by the literature highlighted above, that transit peptides are specific for one or other organelle. Now there are a number of situations in which it is desirable for (often heterologous) proteins of interest to be targeted to both chloroplasts and mitochondria, whether for the purpose of manipulating metabolism in both organelles or for other reasons. Examples include the manipulation of the antioxidant, enzymic or other content of these organelles to increase tolerance to abiotic or environmental stress and the enhancement of resistance to herbicides which interfere with electron transport in both organelles.

At present, the only way in which it is apparent from the art that both organelles can be targeted is to transform the plant with recombinant DNA encoding two fusion proteins: one comprising the protein of interest fused to a chloroplast-specific transit peptide and the other comprising the protein fused to a mitochondrion-specific transit peptide. The plant therefore needs to be made doubly transgenic. This clearly involves significant effort in terms of the initial transformation work and there is always the problem that the two transgenes may

not co-segregate in progeny of the plant, making subsequent breeding from a transgenic parent difficult. Regulatory issues may also militate against doubly transgenic plants.

There is therefore a real need in the art for a transit peptide which co-targets to both the chloroplast and the mitochondrion, to enable a plant to be made singly transgenic for a transgene which encodes a protein which is targeted to both organelles. The present invention is based on the remarkable discovery that one of the transit peptides which was thought to be specific for the chloroplast does in fact meet this need. The transit peptide in question is the glutathione reductase pre-sequence. Neither the Creissen *et al.* paper *supra* nor, it is believed, the rest of the literature actually discloses fusions between the glutathione reductase pre-sequence and a heterologous protein.

According to a first aspect of the invention, there is provided a fusion protein comprising a protein of interest and sufficient of the N-terminal pre-sequence of a glutathione reductase to cause targeting of the protein to both chloroplasts and mitochondria.

The glutathione reductase (GR) which supplies the pre-sequence may be derived from any suitable organism. All that is required is that the organism in question be such that its GR has the ability to co-target both chloroplasts and mitochondria in plants. The GR will usually be derived from a plant, particularly a higher plant such as those of the class *Gymnospermae* or, preferably, *Angiospermae*. Angiosperms of the family *Leguminosae* are preferred, particularly species of the

genus *Pisum*. A highly suitable source of GR is the pea (*Pisum sativum* L.).

It is not necessary for all the natural GR pre-sequence, from whatever source, to be present: only the minimum amount necessary to achieve the targeting (and, if desired, to enable subsequent cleavage) has to be present.

In the case of pea GR, the complete pre-GR sequence is set out in Creissen *et al. supra* and the transit peptide includes at least some of the following residues:

MNQAMATPLS LSCCSPTLTR STLFFTKTFP FSRSFSTPLP

LSTKTLISLS PPHRTFAVRA ESQNGADPAR Q

The natural transit peptide comprises about 60 to 70 residues, all of which may be present in embodiments of the present invention.

It is preferred that at least the following residues be present:

MNQAMATPLS LSCCSPTLTR STLFFTKTFP FSRSFSTPLP

LSTKTLISLS PPHRT

as those residues are not shared with *Ps. aeruginosa*, *E. coli* or human glutathione reductase. The residues FAV may also be present to the C-terminal side of that sequence; and the further residues RAESQNGADPARQ may be further added to the C-terminal side.

While it is expected with the current state of the art that it will be preferred to use transit peptides in the

invention which are identical to natural GR transit peptides (particularly that of *P. sativum*), a degree of divergence from the natural or consensus sequence can be tolerated in the invention provided only that the co-targeting ability of the transit peptide is not lost. Typically, a mutant, variant or derivative transit peptide useful in the invention will be homologous with the natural sequence to the extent of about 60% or even 90% or 95%.

The invention is not limited by the protein of interest which may be targeted to both chloroplasts and mitochondria by means of the invention. Some proteins of interest will be enzymes (even mature glutathione reductase), but whatever their nature their presence will simply be dictated by the particular purpose of the embodiment of the invention in question. Among the preferred purposes of embodiments of the invention are:

1. The manipulation of the antioxidant content of these organelles (eg, glutathione, ascorbate) in order to enhance tolerance to a range of abiotic stresses, thus protecting both major sites of oxygen metabolism in the plant cell (Creissen *et al*, *Proc. Royal Society Edinburgh* (1994));

2. The simultaneous targeting to both compartments of enzymes involved in oxyradical scavenging or antioxidant metabolism e.g. pea Cu/Zn superoxidase (EMBL accession code: PSSOD), pea Mn superoxide dismutase (EMBL accession code: PSSVPOXRE), maize catalase (EMBL accession code: ZMCATZ), pea ascorbate peroxidase (EMBL accession code: PSAPXIA), *E. coli* glutathione reductase (EMBL accession code:

ECSGSHII) and *E. coli* gamma glutanyl cysteine synthetase (EMBL accession code: ECGSHI) for increased scavenging of superoxide leading to enhanced tolerance to environmental stress (see, for example, Bowler *et al.*, *The EMBO Journal* 8(1) 31-38 (1989)); and

3. To enhance tolerance to herbicides which disrupt a critical biochemical process e.g. electron transport in both chloroplasts and mitochondria, by co-targeting resistance genes to these herbicides; for example, resistance to paraquat or acifluorfen.

Fusion proteins of the invention may in principle be made by any convenient process, including *de novo* chemical synthesis. In practice, recombinant DNA technology provides the method of choice, and the fusion proteins will be expressed from a recombinant DNA molecule.

According to a second aspect of the invention, there is provided a recombinant DNA molecule encoding a fusion of a protein of interest with sufficient of the N-terminal pre-sequence of a glutathione reductase to cause targeting of the protein to both chloroplasts and mitochondria.

Recombinant or isolated DNA molecules encoding the transit peptide alone, in the absence of the mature GR protein-coding sequence, are useful for ligation to DNA sequences encoding proteins of interest. According to a third aspect of the invention, therefore, there is provided a recombinant or isolated DNA molecule encoding sufficient of the N-terminal pre-sequence of a glutathione reductase to cause targeting of a protein to

both chloroplasts and mitochondria, provided that in the said isolated or recombinant DNA molecule the DNA encoding the pre-sequence is not precisely fused to DNA encoding mature glutathione reductase.

DNA molecules in accordance with the invention may, if encoding a natural GR transit peptide, correspond to a CDNA or genomic sequence; in other words the presence or absence of any natural introns is not critical to the functioning of the invention, although it may be expected that the presence of one or more natural introns can have implications for expression efficiency.

Recombinant DNA in accordance with the invention may be in the form of a vector. The vector may for example be a plasmid, cosmid or phage. Vectors will frequently include one or more selectable markers to enable selection of cells transfected (or transformed: the terms are used interchangeably in this specification) with them and, preferably, to enable selection of cells harbouring vectors incorporating heterologous DNA. Appropriate start and stop signals will generally be present. Additionally, if the vector is intended for expression, sufficient regulatory sequences to drive expression will be present; however, DNA in accordance with the invention will generally be expressed in cells containing both chloroplasts and mitochondria, e.g. plant cells and algae. Vectors not including microbial regulatory sequences are useful as cloning vectors. For expression in plants, a plant promoter will generally be present operably coupled to sequences to be expressed; any suitable promoter may be used, such as, for example, the 35S Cauliflower Mosaic Virus (CaMV) promoter, the rubisco small subunit (rbs c), a ubiquitin, the plastocyanin or

the *Agrobacterium* nopaline synthase (nos) promoter.

Cloning vectors can be introduced into *E. coli* or another suitable host which facilitate their manipulation. According to a fourth aspect of the invention, there is therefore provided a host cell transfected or transformed with DNA as described above.

DNA in accordance with the invention can be prepared by any convenient method involving coupling together successive nucleotides, and/or ligating oligo- and/or poly-nucleotides, including *in vitro* processes, but again recombinant DNA technology forms the method of choice.

Ultimately, DNA in accordance with the invention will be introduced into plant cells, by any suitable means. According to a fifth aspect of the invention, there is provided a plant cell including DNA in accordance with the invention as described above.

Plants transformed with the DNA segment containing the pre-sequence may be produced by standard techniques which are already known for the genetic manipulation of plants. DNA can be transformed into plant cells using any suitable technology, such as a disarmed Ti-plasmid vector carried by *Agrobacterium* exploiting its natural gene transfer ability (EP-A-270355, EP-A-0116718, Bevan, *Nucleic Acids Research*, **12(22)**: 8711-8721 (1984)). particle or microprojectile bombardment (US-A-5100792, EP-A-444882, EP-A-434616), microinjection (WO 92/09696, WO 94/00583, EP-A-331083, EP-A-175966), electroporation (EP-A-290395, WO-A-8706614). *Agrobacterium* transformation is widely used by those skilled in the art to transform dicotyledonous species. Although

Agrobacterium has been reported to be able to transform foreign DNA into some monocotyledonous species (WO 92/14828), microprojectile bombardments, electroporation and direct uptake are preferred where *Agrobacterium* is inefficient or ineffective. Alternatively, a combination of different techniques may be employed to enhance the efficiency of the transformation process, e.g. bombardment with *Agrobacterium* coated microparticles (EP-A-486234) or microprojectile bombardment to induce wounding followed by co-cultivation with *Agrobacterium* (EP-A-486233).

The particular choice of transformation technology will be determined by its efficiency to transform certain plant species, as well as the experience and preference of the person practising the invention with a particular methodology of choice. It will be apparent to the skilled person that the particular choice of a transformation system to introduce chimeric genes into the plant cells or algae is not essential to the invention.

Alterntively, the foreign DNA could be introduced directly into plant cells using a particle bombardment apparatus. This method is preferred where *Agrobacterium* is ineffective, for example where the recipient plant is monocotyledonous. Any other method that provides for the stable incorporation of the DNA within the nuclear DNA of any plant cell of any species would also be suitable.

DNA in accordance with some embodiments of the invention may also contain a second chimeric gene (a "marker" gene) that enables a transformed plant containing the foreign DNA to be easily distinguished from other plants that do

not contain the foreign DNA. Examples of such a marker gene include antibiotic resistance (Herrera-Estrella *et al*, *The EMBO Journal*, 2 987-995 (1983)), herbicide resistance (EP-A-0242246) and glucuronidase (GUS) expression (EP-A-0344029); however, in some embodiments of the invention the protein of interest may serve as its own marker gene and so no second marker will necessarily be needed. Expression of the marker gene, if present, is preferably controlled by a second promoter (which may also be the 35S CaMV promoter). However any other suitable second promoter could be used.

A whole plant can be regenerated from a single transformed plant cell, and the invention therefore provides in a sixth aspect transgenic plants (or parts of them, such as propagating material) including DNA in accordance with the invention as described above. The regeneration can proceed by known methods.

A singular advantage, quite literally, of the invention is that, as discussed above, a plant does not have to be doubly transgenic if the same protein of interest is to be targeted to both mitochondria and chloroplasts. According to a seventh aspect of the invention, therefore, there is provided a plant having a transgene encoding a fusion of a protein and sufficient of the N-terminal leader sequence of a plant glutathione reductase to cause targeting of the said protein to both mitochondria and chloroplasts, wherein the plant does not have a further transgene which encodes a second mitochondrion- or chloroplast-targeting sequence fused to the said protein.

Transgenic plants in accordance with the invention are

not limited by species. Much work on transgenic plants has been done in tobacco (*Nicotiana tabacum*), which is consequently one of the better understood transgenic hosts (and which is represented in the examples of this invention, shown below). However, the invention is in no sense limited in its usefulness to tobacco or any other individual species.

In general from the above discussions it can be seen that the invention also provides, in an eighth aspect, a method of targeting a protein to both mitochondria and chloroplasts, the method comprising expressing the said protein in a plant as a fusion with sufficient of the N-terminal leader sequence of a plant glutathione reductase to cause targeting of the protein to both organelles.

Preferred features of each aspect of the invention are as for each of the other aspects, *mutatis mutandis*.

The invention will now be illustrated by the following examples. The examples refer to the accompanying drawings, in which:

FIGURE 1 is a map of plasmid pGR46; 'LB' and 'RB' represent the left and right borders, respectively;

FIGURE 2 shows the complete cDNA sequence of pea glutathione reductase, with the deduced amino acid sequence;

FIGURE 3 shows an amino acid alignment of glutathione reductases from pea (Peagr), *Pseudomonas aeruginosa* (Psagr), *Escherichia coli* (Ecgr) and man (Humgr); conserved regions are shaded;

FIGURE 4 is a map of plasmid pGR50; 'LB' and 'RB' represent the left and right borders, respectively; and

FIGURE 5 is a map of plasmid pGR42.

EXAMPLE 1 - Isolation of Glutathione Reductase cDNAs

Glutathione reductase (GR) CDNAS were isolated from a bacteriophage λgt11 cDNA expression library constructed from poly(A)$^+$ RNA isolated from 14 day old pea seedlings. λgt11 is available from Amersham International plc, Amersham and the cDNA library was constructed essentially following the supplier's instructions and as indicated by Creissen *et al.* (*The Plant Journal* **2**(1) 129-131 (1992)). The appropriate recombinant phage were identified by immunodetection using an anti-GR antiserum raised in rabbits against purified pea GR protein (Edwards *et al.*, *Planta* **180** 278-284 (1990)). Immunodetection was achieved by virtue of the recognition by anti-GR of antibodies of the phage-directed synthesis of a β-galactosidase-GR fusion protein. After purification of candidate phage and isolation of their DNA, cDNA inserts were subcloned into the plasmid vector pBLUESCRIPT SKII+ (Stratagene Ltd., Cambridge) as *Bam*HI fragments. DNA sequence analysis was performed using the dideoxy-chain termination procedure (Sanger *et al.*, *Proc. Nat'l. Acad. Sci. USA* **74** 5463-5467 (1977)) on single-stranded and double-stranded DNA templates. The largest cDNA recovered at this stage was termed pGR27 which appeared to encode the mature GR peptide. The cDNA pGR201 was subsequently recovered from the same cDNA library using the 5'-region (co-ordinate 254-393 of the published sequence) of pGR27 as a radiolabelled probe. DNA sequence analysis of the

subcloned cDNA in pGR201 revealed a coding sequence which was clearly identified as GR by amino-acid sequence homology to known GR sequences from other sources (*Homo sapiens*, *Escherichia coli* and *Pseudomonas aeruginosa*). However the pGR201-encoded cDNA also encodes an N-terminal extension, which at the time of first analysis, was determined to be most likely a chloroplast targeting sequence (Creissen *et al*, *supra*). Upstream of the first in-frame methionine initiator codon (AUG) was a translational stop codon. Therefore the cDNA in pGR201 was deemed to encode the full length GR preprotein.

EXAMPLE 2 - Construction of chimeric genes; *in vitro* manipulation of pGR201

As part of a programme for engineering abiotic stress tolerance in plants, GR cDNAs were manipulated *in vitro* to produce the following chimeric genes.

pGR42 was constructed as follows: The *Eco*RV-*Bam*HI fragment (co-ordinates 18-2029) from pGR201 was recovered and inserted into the expression cassette pJIT163-*Bgl*II. pJIT163-*Bgl*II contains 35S promoter and polyadenylation sequences from cauliflower mosaic virus (CaMV) separated by a restriction-site polylinker. pJIT163-*Bgl*II was made from pJIT163 (Guerineau *et al.*, *Plant Molecular Biology*, **18** 815-818 (1992)) by insertion of a *Bgl*II linker (5'-GCAGATCTGC-3') into the *Sac*I site of pJIT163 located at the 5'-end of the 35S promoter.

EXAMPLE 3 - Insertion of chimeric GR gene into a binary Ti vector for *Agrobacterium*-mediated transformation of plants

The chimeric gene of Example 2 above was subcloned as a *Bgl*II fragment into the *Bam*HI site of pBinLuc23. pBinLuc23 comprises the binary Ti vector pBin19 (Bevan, *Nucleic Acids Research*, 12 8711-8721 (1984)) into which a 35S promoter-luciferase gene (Mullineaux *et al.*, *Nucleic Acids Research*, 18 7259-7265 (1990)) was inserted at the *Sac*I site. The chimeric GR gene was inserted between a Kanamycin resistance gene and the 35S-luciferase gene within the T-DNA borders of the vector. The plasmid was designated pGR46 (Figure 1). The plasmid pGR46 was introduced into *Agrobacterium tumefaciens* strain LBA4404 by a triparental mating technique (Ditta *et al*, *Proc. Natl. Acad. Sci.* USA, 77: 7374 (1990)). The *Agrobacterium* containing pGR46 was used to transform tobacco (*Nicotiana tabacum* L. cv. Samsun NN).

EXAMPLE 4 - Transformation of tobacco

Leaf discs of cv. Samsun NN were co-cultivated with *A. tumefaciens* containing the pGR46 essentially as described by Guerineau *et al.*, *Plant Molecular Biology*, 15 127-136 (1990). Potentially transformed shoots were identified as being resistant to 100 mg/l kanamycin sulphate in the growth medium. Putative transformed shoots were rooted on 100 mg/l kanamycin sulphate-containing rooting medium, and confirmed by screening the shoots for luciferase activity as described by Mullineaux *et al.* (1990) *supra*. Kanamycin resistant, luciferase positive (Kan^r, luc^+) plantlets were potted in soil and grown to maturity in the glasshouse. Seed was collected from self-pollinated

plants (T_1 progeny).

EXAMPLE 5 - Compartmentation analysis

T_1 progeny from 5 independently transformed pGR46 lines were germinated and screened for segregation of luciferase activity (ie luc^+luc^-). GR activity was assayed (Edwards *et al.*, *Planta*, **180** 278-284 (1990)) from total pooled extracts of each line of luc^+ seedlings was determined (Table 1).

Table 1

LINE	GR ACTIVITY (nmols NADPH oxidised min^{-1} mg protein)	INCREASE OVER CONTROL (fold)
control tobacco	6	1
46-19	102	17
46-20	205	34
46-23	88	15
46-27	124	21
46-29	123	20

Two independently transformed lines were selected for further analysis. These were 46-23 and 46-27. Enhanced synthesis of GR was determined by immunodetection on Western blots. Luc^+ and luc^- T_1 plants from each of these lines were used as sources of chloroplast and mitochondria.

Chloroplast fractionation was carried out using the method of Boutry *et al.*, *Nature*, **328** 340-342 (1987) with the following modifications:

1. The plant material was homogenised with a Polytron.
2. 1 mM EDTA was added to the grinding medium.
3. Percoll was diluted in the grinding medium, rather

than second suspension medium.

4. After recovery of purified chloroplast fraction from Percoll gradients, the chloroplasts were washed in 10 volumes of HEPES-sorbitol medium (50 mM HEPES, 330 mM sorbitol, 10 mM NaCl, 1 mM $MgCl_2$, 2mM KH_2PO_4, pH7.6) and resuspended in the same medium.

5. Chloroplasts were lysed for enzyme assays by adding an equal volume of lysis buffer (50 mM Tris-HCl pH7.5, 5 mM $MgCl_2$, 2 mM dithiothreitol, 1 mM EDTA, 0.2% Triton X-100).

To confirm the purity of chloroplast fractions, enzymes were assayed which are specific to each of the major cell compartments (Edwards *et al.*, *Planta*, **180** 278-284 (1990)). These are glyceraldehyde 3-phosphate dehydrogenase (GAPDH; chloroplast; Wu and Racker, *J. Biological Chemistry*, **234** 1029-1035 (1959)); cytochrome C oxidase (CCO; mitochondria; Tolbert, *Methods of Enzymology*, **31A** 734-746 (1974)) and pyrophosphate-dependent phosphofructokinase (PPi-PFK; cytosol; Journet and Douce, *Plant Physiology*, 79 458-467 (1985)). Data from two experiments, one on each of the selected lines, are presented in Table 2.

Table 2

LINE/FRACTION	GR	GAPDH	CCO	PPi-PFK
46-27, luc$^+$: total	220	80	N/A	40
46-27, luc$^-$: total	10	50	N/A	20
46-23, luc$^+$: total	100	20	N/A	20
46-23, luc$^-$: total	20	30	N/A	30
46-27, luc$^+$: chloroplast	130	30	0	0
46-27, luc$^-$: chloroplast	8	20	0	0
46-23, luc$^+$: chloroplast	70	30	0	0
46-23, luc$^-$: chloroplast	10	30	0	0

NOTES One unit of each of GR and GAPDH activity represents one nmol NADPH oxidised per minute per mg protein. One unit of CCO activity represents one µmol reduced cytochrome C oxidised per minute per mg protein. One unit of PPi-PFK activity represents one nmol NADH oxidised per minute per mg protein. 'N/A' means not assayed.

The data clearly show the elevation of GR activities in chloroplasts of luc$^+$ plants compared with their luc$^-$ siblings, confirming that the product of the pGR201 cDNA is targeted to the chloroplast. Mitochondrion fractionation was carried out using the method of Boutry *et al* (1987) *supra* as described for chloroplasts with the following modifications:

1. Ascorbic acid was omitted from the grinding medium; and
2. Mitochondria were layered onto a single concentration of Percoll (50% v/v) in grinding medium.

Other than these two specific modifications for mitochondria, all other modifications to the method of Boutry *et al*, as described for chloroplasts, are used for

the mitochondrion preparation.

The marker enzymes used to establish the purity of the mitochondrion preparation were the same as those used for the chloroplast preparation. Data from two experiments, one on each of the selected lines, are presented in Table 3.

Table 3

LINE/FRACTION	GR	GAPDH	CCO	PPi-PFK
46-27, luc$^+$: total	100	80	27	20
46-27, luc$^-$: total	20	120	24	20
46-23, luc$^+$: total	90	70	23	10
46-23, luc$^-$: total	20	90	14	10
46-27, luc$^+$: mitochondrion	50	0	1895	0
46-27, luc$^-$: mitochondrion	0	0	1895	0
46-23, luc$^+$: mitochondrion	80(*)	20	345	0
46-23, luc$^-$: mitochondrion	0	10	403	0

NOTES One unit of each of GR and GAPDH activity represents one nmol NADPH oxidised per minute per mg protein. One unit of CCO activity represents one µmol reduced cytochrome C oxidised per minute per mg protein. One unit of PPi-PFK activity represents one nmol NADH oxidised per minute per mg protein. (*) = 3% of this GR activity is attributable to chloroplast contamination as indicated by the presence of GAPDH activity in the mitochondrial fraction.

The data clearly show the elevation of GR activities in mitochondria of luc$^+$ plants compared with their luc$^-$ siblings, showing that the product of the pGR201 cDNA is also targeted to the mitochondrion.

Therefore, taking the data presented in Tables 2 and 3 together, it is clear that the cDNA pGR201 can direct GR to both of these organelles.

EXAMPLE 6 - Construction of a chimeric gene consisting of a fusion between the sequences encoding the amino terminus of pea glutathione reductase and the coding sequence for phosphinothricin acetyl transferase from *Streptomyces hygroscopicus*

Starting materials:

The source of the glutathione reductase (GR) sequence was the plasmid pGR42 (see Example 2). This contains the pea glutathione reductase cDNA clone (Creissen *et al*, 1992, supra) under the control of the CaMV 35S promoter and polyadenylation sequences.

The phosphinothricin acetyl transferase (*pat*) coding sequence was obtained from the plasmid pIJ4102. pIJ4102 is identical to plasmid pIJ4104 described by White *et al*, *Nucl. Acids Res.*, **18**: 1063 (1990).

Description of the chimeric GR:*pat* gene construct:

The chimeric gene construct comprised the CaMV 35S promoter with duplicated enhancer region and CaMV polyadenylation signals (Guerineau *et al*, 1992, supra), flanking a fusion between the 5'-region of the pea GR cDNA (pGR201; co-ordinates 18-392) and the *pat* coding sequence such that translation would be initiated at one of the GR AUG codons and continue to the translational stop codon at the 3'-end of the *pat* coding sequence. The *pat* coding sequence data is lodged with the EMBL database as entries SHBRPA and X17220.

Cloning Strategy:

1. The *pat* coding sequence was released from pIJ4102 by digestion with XhoI, followed by treatment with bacteriophage T4 DNA polymerase and subsequent digestion with BglII. The ca. 550bp *pat* coding sequence was eluted from an agarose gel.

2. The plasmid pGR42 was digested with SacI, followed by treatment with bacteriophage T4 DNA polymerase and subsequent digestion with BamHI. The fragment comprising the vector plus CaMV promoter and polyadenylation sequences and 5' end of GR was eluted from an agarose gel.

3. The chimeric expression cassette pGR48 was generated by ligation of the two fragments from 1 and 2 above.

4. Finally, the chimeric gene was excised as a BglII fragment and ligated into the unique BamHI site of the binary vector pBINLUC23 (see Example 3) to create pGR50.

Transformation of tobacco:

The plasmid pGR50 was mobilised into *Agrobacterium tumefaciens* LBA4404 by the triparental mating procedure (Ditta *et al*, *Proc. Natl. Acad. Sci. USA*. **77**: 7374 (1990)) and used to transform tobacco by leaf disc co-cultivation (Horsch *et al*, *Science*, **223**: 496 (1984)). Putative transgenic plants were identified by their ability to root on kanamycin-containing medium. Kanamycin-resistant shoots, which were found also to be expressing the firefly luciferase (T-DNA right border

marker), were transferred to the glasshouse and seeds were collected from self-pollinated plants.

Seeds were sown on phosphinothricin-containing medium (10 ug/ml) and were found to exhibit the predicted 3:1 segregation of resistance, confirming that there was a single locus for the T-DNA and that the fusion protein expressed from the chimeric gene was biologically active. The presence of the PAT protein in chloroplasts and mitochondria are confirmed using the methods detailed above (see Example 5).

CLAIMS

1. A fusion protein comprising a protein of interest and sufficient of the N-terminal pre-sequence of a glutathione reductase to cause targeting of the protein to both chloroplasts and mitochondria.

2. A fusion protein as claimed in claim 1, wherein the source of glutathione reductase is the pea (*Pisum sativum* L.).

3. A fusion protein as claimed in claim 1 or 2, wherein the pre-sequence includes at least some of the following residues:

MNQAMATPLS LSCCSPTLTR STLFFTKTFP FSRSFSTPLP LSTKTLISLS PPHRTFAVRA ESQNGADPAR Q.

4. A fusion protein as claimed in claim 3, including at least the following residues:

MNQAMATPLS LSCCSPTLTR STLFFTKTFP FSRSFSTPLP LSTKTLISLS PPHRT.

5. A fusion protein as claimed in claim 4, further including the residues FAV to the C-terminal side of the N-terminal pre-sequence.

6. A fusion protein as claimed in claim 5, further including the residues RAESQNGADPARQ to the C-terminal side of the N-terminal pre-sequence.

7. A fusion protein as claimed in any one of claims 1

to 6, wherein the protein of interest is implicated in the manipulation of the antioxidant content.

8. A fusion protein as claimed in any one of claims 1 to 6, wherein the protein of interest is an enzyme involved in oxyradical scavenging or antioxidant metabolism.

9. A fusion protein as claimed in claim 8 wherein the protein of interest is superoxide dismutase.

10. A fusion protein as claimed in any one of claims 1 to 6, wherein the protein of interest is implicated in the enhancement of tolerance to herbicides which disrupt a critical biochemical process in either chloroplasts or mitochondria or both.

11. A fusion protein as claimed in claim 10 wherein the critical biochemical process is electron transport.

12. A recombinant DNA molecule encoding a fusion of a protein of interest with sufficient of the N-terminal pre-sequence of a glutathione reductase to cause targeting of the protein to both chloroplasts and mitochondria.

13. A DNA molecule as claimed in claim 12 which encodes a fusion protein as claimed in any one of claims 2 to 11.

14. A recombinant or isolated DNA molecule encoding sufficient of the N-terminal pre-sequence of a glutathione reductase to cause targeting of a protein to both chloroplasts and mitochondria, provided that in the said isolated or recombinant DNA molecule the DNA encoding the pre-sequence is not precisely fused to DNA

encoding mature glutathione reductase.

15. DNA as claimed in claim 12, 13 or 14, which is in the form of a vector.

16. DNA as claimed in any one of claims 12 to 15, including a functional promoter, operative in a plant.

17. DNA as claimed in claim 16, wherein the promoter is the 35S Cauliflower Mosaic Virus (CaMV) promoter.

18. A host cell transfected or transformed with DNA as claimed in any one of claims 12 to 17.

19. A plant cell including DNA as claimed in any one of claims 12 to 17.

20. A transgenic plant (or parts of a transgenic plant) including DNA as claimed in any one of claims 12 to 17.

21. A plant having a transgene encoding a fusion ofa protein and sufficient of the N-terminal leader sequence of a plant glutathione reductase to cause targeting of the said protein to both mitochondria and chloroplasts, wherein the plant does not have a further transgene which encodes a second mitochondrion- or chloroplast-targeting sequence fused to the said protein.

22. A method of targeting a protein to both mitochondria and chloroplasts, the method comprising expressing the said protein in a plant as a fusion with sufficient of the N-terminal leader sequence of a plant glutathione reductase to cause targeting of the protein to both organelles.

FIG. 1

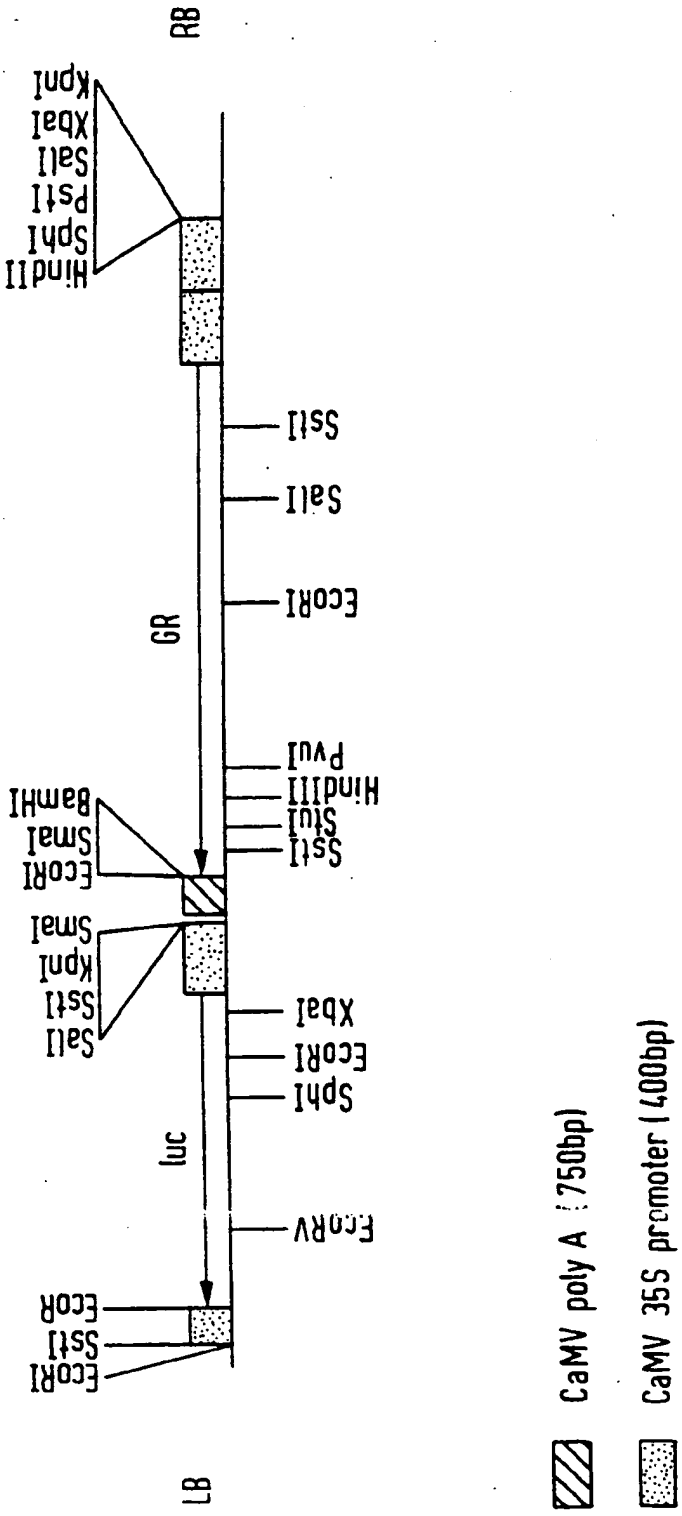

RB
KpnI
XbaI
SalI
PstI
SphI
HindII
SstI
SalI
EcoRI
GR
PvuI
HindIII
StuI
SstI
BamHI
SmaI
EcoRI
SmaI
KpnI
SstI
SalI
XbaI
EcoRI
SphI
luc
EcoRV
EcoR
SstI
EcoRI
LB
CaMV poly A (750bp)
CaMV 35S promoter (400bp)

FIGURE 2

```
1    CTTGTATTACTCCACCGAGATATCCGGATTATCAAGGTGGTTGGTTACTAAGCACCACCG
       L  V  L  L  H  R  D  I  R  I  I  K  V  V  G  Y  *  A  P  P -

61   GGAACAGAGAGGGAAACTCTAATGAACCAAGCAATGGCTACTCCACTTTCTCTCTCTTGT
       G  T  E  R  E  T  L  M  N  Q  A  M  A  T  P  L  S  L  S  C -

121  TGTTCTCCAACTCTAACTCGCAGCACTCTCTTCTTCACCAAAACCTTTCCATTTTCTCGC
       C  S  P  T  L  T  R  S  T  L  F  F  T  K  T  F  P  F  S  R -

181  TCCTTCTCTACACCTCTCCCTCTCTCCACCAAAACCCTAATTTCCCTCTCTCCACCCCAT
       S  F  S  T  P  L  P  L  S  T  K  T  L  I  S  L  S  P  P  H -

241  CGCACCTTCGCCGTCCGCGCTGAATCCCAAAACGGCGCCGACCCCGCCCGCCAGTATGAC
       R  T  F  A  V  R  A  E  S  Q  N  G  A  D  P  A  R  Q  Y  D -

301  TTCGACCTTTTCACCATCGGCGCTGGAAGCGGAGGCGTCCGTGCTTCCCGCTTTGCCTCC
       F  D  L  F  T  I  G  A  G  S  G  G  V  R  A  S  R  F  A  S -

361  AATTTCGGCGCTTCTTCTGCTGTCTGCGAGCTCCCTTTCTCTACTATCTCCTCCGATACC
       N  F  G  A  S  S  A  V  C  E  L  P  F  S  T  I  S  S  D  T -

421  ACCGGTGGTGTCGGCGGCACCTGTGTAATACGGGGATGTGTCCCTAAGAAATTGCTAGTC
       T  G  G  V  G  G  T  C  V  I  R  G  C  V  P  K  K  L  L  V -

481  TATGCCTCAAAATTCTCTCATGAATTTGAAGAAAGCAATGGTTTTGGATGGAGATATGAC
       Y  A  S  K  F  S  H  E  F  E  E  S  N  G  F  G  W  R  Y  D -

541  AGTGAACCTAAGCATGACTGGAGTAGTTTGATTGCTAATAAAAACGCCGAGTTGCAGCGG
       S  E  P  K  H  D  W  S  S  L  I  A  N  K  N  A  E  L  Q  R -

601  CTTACTGGTATCTATAAGAATACTTTGAAAAATGCCGGTGTTAAGTTGATTGAAGGCCGT
       L  T  G  I  Y  K  N  T  L  K  N  A  G  V  K  L  I  E  G  R -

661  GGAAAGATTGTAGATGCTCACACAGTTGATGTTGATGGGAAGTTATATTCAGCGAAACAC
       G  K  I  V  D  A  H  T  V  D  V  D  G  K  L  Y  S  A  K  H -

721  ATTTTAGTTTCAGTTGGAGGTCGACCCTTCATTCCTGATATTCCTGGAAAGGAATATGCA
       I  L  V  S  V  G  G  R  P  F  I  P  D  I  P  G  K  E  Y  A -

781  ATAGATTCAGATGCTGCCCTTGATTTACCATCAAAGCCTCAGAAGATAGCTATTGTTGGT
       I  D  S  D  A  A  L  D  L  P  S  K  P  Q  K  I  A  I  V  G -

841  GGGGGTTACATTGCCTTGGAGTTTGCTGGTATCTTTAATGGTTTGAAAAGTGAAGTTCAT
       G  G  Y  I  A  L  E  F  A  G  I  F  N  G  L  K  S  E  V  H -

901  GTATTTATAAGACAAAAGAAGGTTTTGCGGGGATTTGATGAAGAGATTAGAGATTTTGTT
       V  F  I  R  Q  K  K  V  L  R  G  F  D  E  E  I  R  D  F  V -

961  GCAGAAAATATGGCTCTGAGAGGTATTGAATTCCATACTGAGGAGTCTCCTGTAGCTATC
       A  E  N  M  A  L  R  G  I  E  F  H  T  E  E  S  P  V  A  I -

1021 ACTAAGGCAGCTGATGGTTCGCTCTCTTTAAAGACCAACAAAGGTACTGAGGAAGGTTTC
       T  K  A  A  D  G  S  L  S  L  K  T  N  K  G  T  E  E  G  F -
```

FIGURE 2 (Cont.)

```
1081 TCTCATATTATGTTTGCCACTGGACGCTCACCTAATACTAAGGATTTGGGCCTGGAGTCT
     S  H  I  M  F  A  T  G  R  S  P  N  T  K  D  L  G  L  E  S  -

1141 GTTGGTGTGAAAGTGGCTAAAGATGGATCAATAGAGGTTGATGAATACTCTCAAACATCG
     V  G  V  K  V  A  K  D  G  S  I  E  V  D  E  Y  S  Q  T  S  -

1201 GTTCCTTCTATTTGGGCAATTGGAGATGCTACAAATAGAGTAAATCTCACTCCAGTTGCT
     V  P  S  I  W  A  I  G  D  A  T  N  R  V  N  L  T  P  V  A  -

1261 TTGATGGAGGGAGTGGCATTAGCAAAAACTTTGTTTCAGAATGAGCCGACAAAACCTGAT
     L  M  E  G  V  A  L  A  K  T  L  F  Q  N  E  P  T  K  P  D  -

1321 TATAGGGCTATACCTTCTGCTGTGTTTTCCCAACCACCAATTGGAGGAGTTGGTCTTACA
     Y  R  A  I  P  S  A  V  F  S  Q  P  P  I  G  G  V  G  L  T  -

1381 GAGGAACAGGCTGCTGAACAATATGGTGATATTGACGTTTTCACAGCAAATTTTAGGCCG
     E  E  Q  A  A  E  Q  Y  G  D  I  D  V  F  T  A  N  F  R  P  -

1441 ATGAAGGCCACCCTCTCTGGGCTTCCAGACCGGGTTTTTATGAAACTAATAGTCTCTGCA
     M  K  A  T  L  S  G  L  P  D  R  V  F  M  K  L  I  V  S  A  -

1501 GAAACAAATGTTGTTCTTGGATTGCACATGTGTGGAGAAGATGCTGCTGAAATTGCACAG
     E  T  N  V  V  L  G  L  H  M  C  G  E  D  A  A  E  I  A  Q  -

1561 GGGTTTGCAGTTGGTATTAAAGCTGGATTAACAAAGGCGGACTTTGATGCCACAGTAGGC
     G  F  A  V  G  I  K  A  G  L  T  K  A  D  F  D  A  T  V  G  -

1621 ATTCATCCAACTGCAGCTGAGGAATTTGTTACCATGAGGACTCCCACTAGGAAGGTTCGA
     I  H  P  T  A  A  E  E  F  V  T  M  R  T  P  T  R  K  V  R  -

1681 AAGAACCAAGCTTCACAGGGGAAGTCAGATTCTAAAGCAAAAGCTGTGGCTGGATCTTAA
     K  N  Q  A  S  Q  G  K  S  D  S  K  A  K  A  V  A  G  S  *  -

1741 GAGTATTAATTTGCTTCAATTATTATACCCAAAGAAACTTGCTGAGGCCTTAAGGCAGGT
     E  Y  *  F  A  S  I  I  I  P  K  E  T  C  *  G  L  K  A  G  -

1801 TATTGAGTTTTCGAGTGATCTCTGTCAACGGAGCTTTCAAGACAATTCATGAAATAGCCT
     Y  *  V  F  E  *  S  L  S  T  E  L  S  R  Q  F  M  K  *  P  -

1861 GCAGAGCTCATCTGGAAAAGGGGAGCAGTGGAATTTTGCGAGCTATTATGTGCAATTTGT
     A  E  L  I  W  K  R  G  A  V  E  F  C  E  L  L  C  A  I  C  -

1921 AATTTATTTCTCACCTTTTTTACCAATTTATTTTTCACCCTAACCTTACCCATTTGTACA
     N  L  F  L  T  F  F  T  N  L  F  F  T  L  T  L  P  I  C  T  -

1981 TATTAAGATGAAATTTCGCGAGGTACTTTGATGTTAAATAAAATAATCT
     Y  *  D  E  I  S  R  G  T  L  M  L  N  K  I  I  -
```

FIGURE 3

```
            1                                                   50
Peagr   APPGTERETL MNQAMATPLS LSCCSPTLTR STLFFTKTFP FSRSFSTPLP

           51                                                   97
Peagr   LSTKTLISLS PPHRTFAVRA ESQNGADPAR Q...YDFDLF TIGAGSGGVR
Psagr                                      MSFDFDLF VIGAGSGGVR
 Ecgr                                      MTKHYDYI AIGGGSGGIA
Humgr                  MACRQ EPQPQGPPPA AGAVASYDYL VIGGGSGGLA
                                                    .....
           98                                                  147
Peagr   ASRFASNFGA SSAVCELPFS TISSDTTGGV GGTCVIRGCV PKKLLVYASK
Psagr   AARFAAGFGA RVAVAESRY. .........L GGTCVNVGCV PKKLLVYGAH
 Ecgr   SINRAAMYGQ KCALIEAKE. .........L GGTCVNVGCV PKKVMWHAAQ
Humgr   SARRAAELGA RAAVVESHK. .........L GGTCVNVGCV PKKVMWNTAV
                                          ........ ...
          148                                                  196
Peagr   FSHEFEE.SN GFGWRYDSEP KHDWSSLIAN KNAELQRLTG IYKNTLKNAG
Psagr   FSEDFEQ.AR AYGWS.AGEA QFDWATLIGN KNREIQRLNG IYRNLLVNSG
 Ecgr   IREAIHMYGP DYGFD.TTIN KFNWETLIAS RTAYIDRIHT SYENVLGKNN
Humgr   HSEFMHD.HA DYGFP.SCEG KFNWRVIKEK RDAYVSRLNA IYQNNLTKSH
                             ^                       ........ ....

          197                                                  241
Peagr   VKLIEGRGKI VDA..HTVDV DGKLYSAKHI LVSVGGRPFI P...DIPGKE
Psagr   VTLLEGHARL LDA..HSVEV DGQRFSAKHI LVATGGWPQV P...DIPGKE
 Ecgr   VDVIKGFARF VDA..KTLEV NGETITADHI LIATGGRPSH P...DIPGVE
Humgr   IEIIRGHAAF TSDPKPTIEV SGKKYTAPHI LIATGGMPST PHESQIPGAS

          242                                                  291
Peagr   YAIDSDAALD LPSKPQKIAI VGGGYIALEF AGIFNGLKSE VHVFIRQKKV
Psagr   HAITSNEAFF LERLPRRVLV VGGGYIAVEF ASIFNGLGAE TTLLYRRDLF
Ecgr    YGIDSDGFFA LPALPERVAV VGAGYIAVEL AGVINGLGAK THLFVRKHAP
Humgr   LGITSDGFFQ LEELPGRSVI VGAGYIAVEM AGILSALGSK TSLMIRHDKV
                                                        *
          292                                                  341
Peagr   LRGFDEEIRD FVAENMALRG IEFHTEESPV AITKAADGSL SLKTNKGTEE
Psagr   LRGFDRSVRE HLRDELGKKG LDLQFNSDIA RIDKQADGSL AATLKDGRVL
 Ecgr   LRSFDPMISE TLVEVMNAEG PQLHTNAIPK AVVKNTDGSL TLELEDGRSE
Humgr   LRSFDSMIST NCTEELENAG VEVLKFSQVK E.VKKTLSGL EVSMVTAVPG
          *
                  342                                          383
Peagr   .........GF SHIMFATGRS PNTKDLGLES VGVKVAKDGS IEVDEYSQTS
Psagr   .........EA DCVFYATGRR PMLDDLGLEN TAVKLTDKGF IAVDEHYQTS
 Ecgr   .........TV DCLIWAIGRE PANDNINLEA AGVKTNEKGY IVVDKYQNTN
Humgr   RLPVMTMIDV DCLLWAIGRV PNTKDLSLNK LGIQTDDKGH IIVDEFQNTN
          384                                                  432
Peagr   VPSIWAIGDA TNRVNLTPVA LMEGVALAKT LFQNEPTKP. DYRAIPSAVF
Psagr   EPSILALGDV IGRVQLTPVA LAEGMAVARR LFKPEEYRPV DYKLIPTAVF
 Ecgr   IEGIYAVGDN TGAVELTPVA VAAGRRLSER LFNNKPDEHL DYSNIPTVVF
Humgr   VKGIYAVGDV CGKALLTPVA IAAGRKLAHR LFEYKEDSKL DYNNIPTVVF
                             .... .......
```

FIGURE 3 (Cont.)

```
          433                                                      480
Peagr     SQPPIGGVGL TEEQAAEQYG D..IDVFTAN FRPMKATLSG LPDRVFMKLI
Psagr     SLPNIGTVGL TEEEALS..A GHKVKIFESR FRPMKLTLTD DQEKTLMKLV
Ecgr      SHPPIGTVGL TEPQAREQYG DDQVKVYKSS FTAMYTAVTT HRQPCRMKLV
Humgr     SHPPIGTVGL TEDEAIHKYG IENVKTYSTS FTPMYHAVTK RKTKCVMKMV
                                  ....
          481                                                      530
Peagr     VSAETNVVLG LHMCGEDAAE IAQGFAVGIK AGLTKADFDA TVGIHPTAAE
Psagr     VDAHDDRVLG CHMVGAEAGE ILQGIAVAMK AGATKQAFDE TIGIHPTAAE
Ecgr      CVGSEEKIVG IHGIGFGMDE MLQGFAVALK MGATKKDFDN TVAIHPTAAE
Humgr     CANKEEKVVG IHMQGLGCDE MLQGFAVAVK MGATKADFDN TVAIHPTSSE
                                                           ......
          531                                 562
Peagr     EFVTMRTPTR KVRKNQASQG KSDSKAKAVA GS
Psagr     EFVTLRTPTR

Ecgr      EFVTMR

Humgr     ELVTLR
          ....
```

FIGURE 4

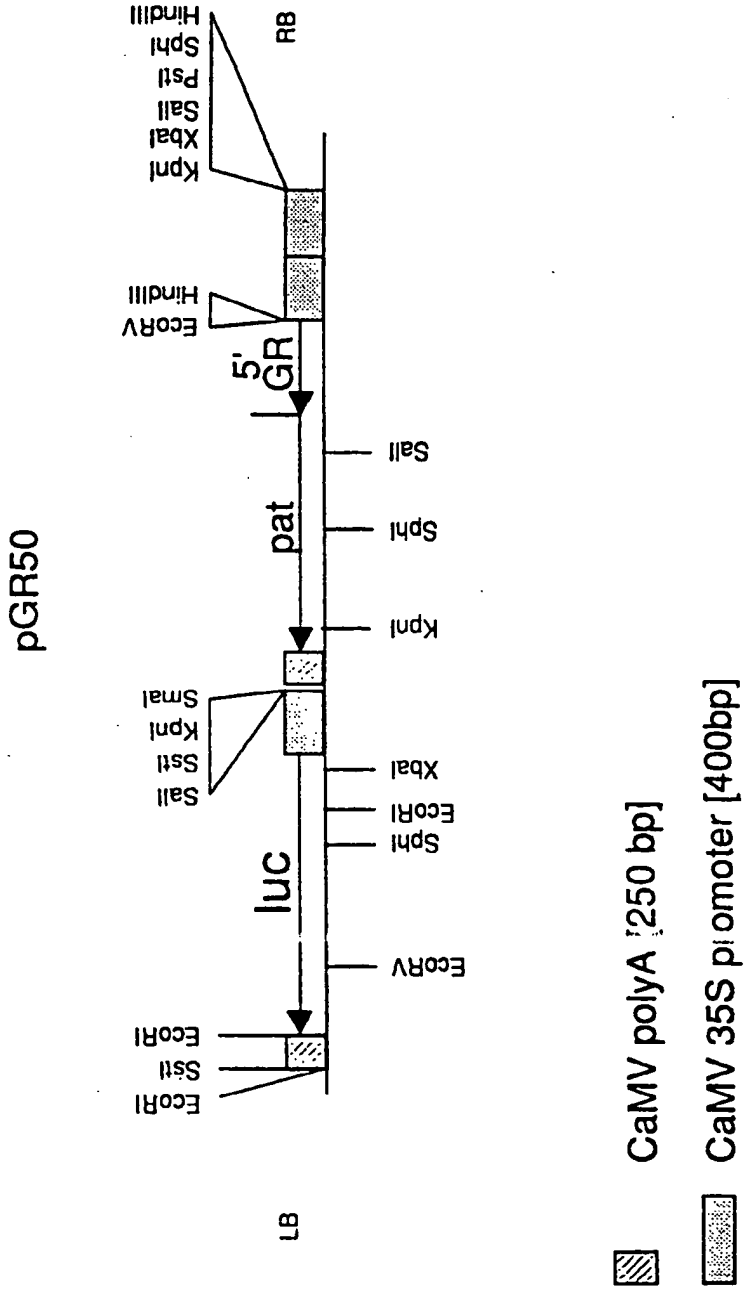
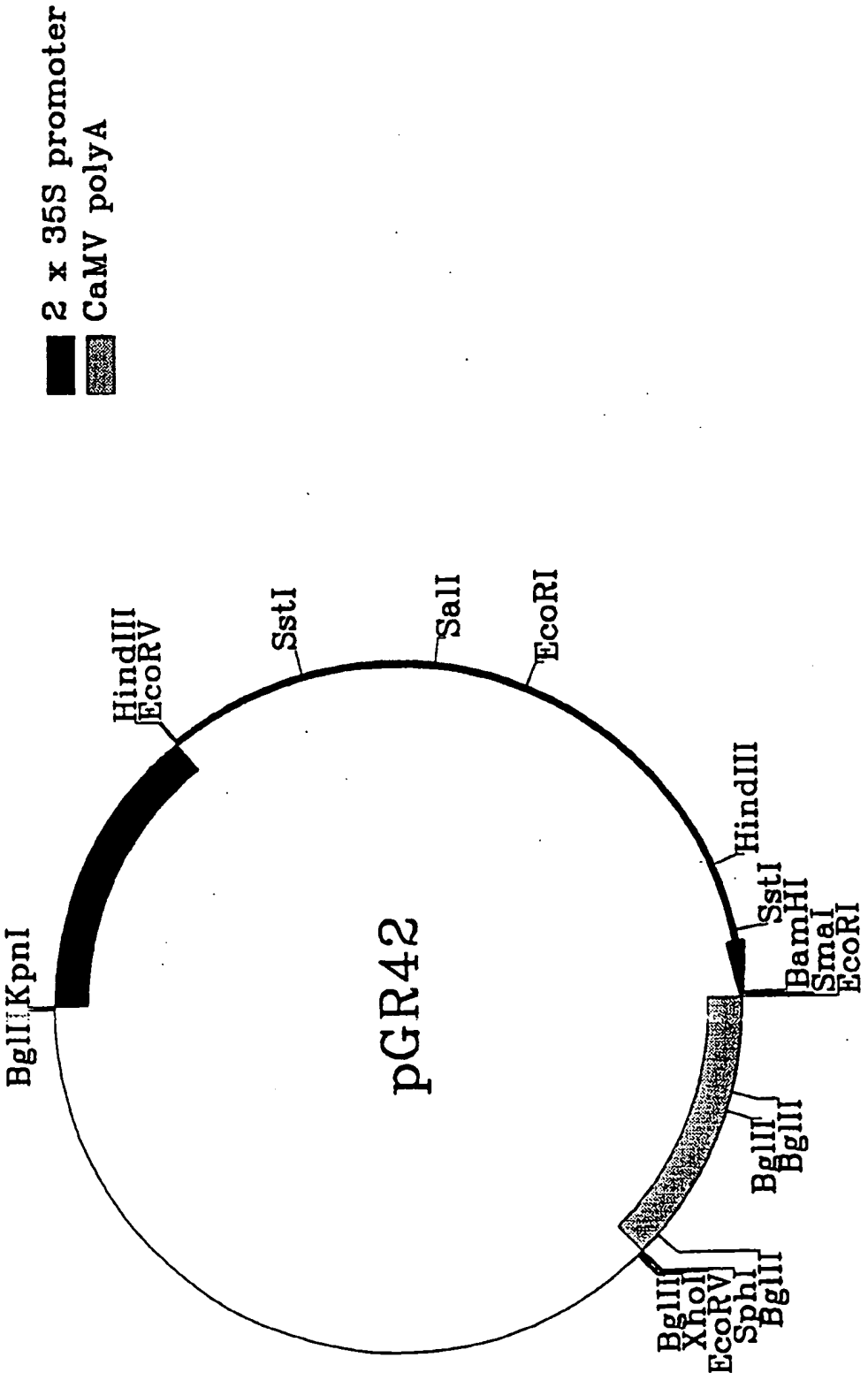

pGR50
RB
HindIII
SphI
PstI
SalI
XbaI
KpnI
EcoRV
HindIII
5' GR
SalI
pat
SphI
KpnI
SmaI
KpnI
SstI
SalI
XbaI
EcoRI
SphI
luc
EcoRV
EcoRI
SstI
EcoRI
LB
CaMV polyA [250 bp]
CaMV 35S promoter [400bp]

FIGURE 5


2 x 35S promoter
CaMV polyA
pGR42
BglII
KpnI
HindIII
EcoRV
SstI
SalI
EcoRI
HindIII
SstI
BamHI
SmaI
EcoRI
BglII
BglII
BglII
XhoI
EcoRV
SphI
BglII

INTERNATIONAL SEARCH REPORT

Intx _ onal Application No
PCT/GB 94/02058

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/53 C12N15/82 C12N15/62 C12N5/10 A01H5/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N A01H

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	THE PLANT JOURNAL, vol.2, no.1, January 1992 pages 129 - 131 CREISSEN, G., ET AL. 'Molecular characterization of glutathione reductase cDNAs from pea (Pisum sativum L.)' cited in the application see the whole document --- -/--	1-22

[X] Further documents are listed in the continuation of box C.

[ ] Patent family members are listed in annex.

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"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

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"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
2 February 1995	10.02.95

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax (+ 31-70) 340-3016	Maddox, A

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	BIOLOGICAL ABSTRACTS, vol. 95 1 May 1993, Philadelphia, PA, US; abstract no. 101542, AONO, M., ET AL. 'Enhanced tolerance to photooxidative stress of transgenic Nicotiana tabacum with high chloroplastic glutathione reductase activity' see abstract & PLANT CELL PHYSIOL., vol.34, no.1, 1993 pages 129 - 135	1-22
A	THE PLANT CELL, vol.2, December 1990 pages 1249 - 1260 HUANG, J., 'A yeast mitochondrial leader peptide functions in vivo as a dual targeting signal for both chloroplasts and mitochondria' see the whole document	1-22